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The *Rhizobium*-Plant Symbiosis

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INTRODUCTION

Under conditions of nitrogen limitation, *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, collectively referred to as rhizobia, elicit on their leguminous hosts the formation of specialized organs, nodules. In these root or stem structures, the bacteria are able to convert atmospheric nitrogen into ammonia, which is used by the plant as a nitrogen source.

Diversity of Legumes

The ability to establish a nitrogen-fixing symbiosis with rhizobia is restricted to legumes, with one exception, the genus *Parasponia* of the Ulmaceae (262). The Leguminosae family comprises three subfamilies, Caesalpinioideae, Mimosoideae, and Papilionoideae, each of which contains genera able to form root nodules (2, 190). In these three subfamilies, the percentages of nodulated species are quite different. There are just a few nonnodulating genera in the advanced subfamilies Papilionoideae and the Mimosoideae, but the less specialized subfamily Caesalpinioideae includes many nonnodulating genera (45). Since the Caesalpinioideae is the most primitive subfamily of the Fabales, it is assumed that the symbiosis was developed at a relatively late stage during legume evolution (288). This notion is also supported by the fact that several *Parasponia* species in the family Ulmaceae form legume-like nitrogen-fixing nodules with a variety of rhizobial strains which can also nodulate some legumes. Since the members of the Ulmaceae are no more closely related to the members of the Leguminosae than to almost any other family of dicotyledons, no real phylogenetic sense can be made of this phenomenon (288). Leguminous plants are very diverse in morphology, habitat, and ecology, ranging from Arctic annuals to tropical trees (45). Because a large number of legumes are nodulated by rhizobia, the symbiosis with rhizobia is apparently not an adaptation to a specialized ecological niche but, rather, depends on some genetic peculiarity of legumes, one that is so complex that it has rarely evolved elsewhere in the plant kingdom (288).

Taxonomy and Host Specificity of *Rhizobium* Species

The three rhizobial genera, *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, have for many years been grouped with the agrobacteria and phyllobacteria into one family, the *Rhizobiaceae* (127). The use of modern methods of bacterial systematics, such as numerical taxonomy, nucleic acid hybridization, and 16S rRNA analysis, has demonstrated the existence of marked genetic diversity within this family (287). It is now widely accepted that *Rhizobium* and *Bradyrhizobium* are only distantly related (167). Each of these genera has close relatives that are not plant symbionts and are placed in different families. This is most evident for *Bradyrhizobium* (283, 285, 287).

The current taxonomic classification of the rhizobia is given

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TABLE 1. Rhizobium-plant associations

Rhizobium	Host plant(s)
R. meliloti	*Medicago*, *Melilotus*, and *Trigonella* spp.
R. leguminosarum	
bv. viciae	*Pisum*, *Vicia*, *Lathyrus*, and *Lens* spp.
bv. trifolii	*Trifolium* spp.
bv. phaseoli	*Phaseolus vulgaris*
R. loti	*Lotus* spp.
R. huakuii	*Astragalus sinicus*
R. ciceri	*Cicer arietinum*
Rhizobium sp. strain NGR234	Tropical legumes, *Parasponia* spp. (nonlegume)
R. tropici	*Phaseolus vulgaris*, *Leucaena* spp., *Macroptilium* spp.
R. etli	*Phaseolus vulgaris*
R. galegae	*Galega officinalis*, *G. orientalis*
R. fredii	*Glycine max*, *G. soja*, and other legumes
B. japonicum	*Glycine max*, *G. soja*, and other legumes
B. elkanii	*Glycine max*, *G. soja*, and other legumes
Bradyrhizobium sp. strain *Parasponia*	*Parasponia* spp.
A. caulinodans	*Sesbania* spp. (stem nodulating)

in Table 1. The species name of the microsymbionts reflects in most cases the corresponding host plant nodulated and suggests that symbiosis is a species-specific process. The situation is much more complex than can be reflected in Table 1, in which some of the host plants are matched up with the microsymbiont. It is quite clear that the degree of host specificity varies tremendously among the rhizobia (288). Some strains have a very narrow host range, for example *Rhizobium leguminosarum* bv. trifolii, while others, like *Rhizobium* sp. strain NGR234, have a very broad host range. It is becoming increasingly clear that the symbiotic relationships between legume species and rhizobia is quite complex, as illustrated by the following examples. *Rhizobium* sp. strain NGR234 nodulates at least 35 different legume genera as well as the nonlegume *Parasponia* (153). Recently, it was demonstrated that *Azorhizobium caulinodans* can nodulate *Phaseolus vulgaris* (280). According to the data in Table 1, rhizobia isolated from alfalfa should belong to the species *R. meliloti*. However, Eardly et al. (67) have shown that some rhizobia isolated from alfalfa, including strain OR191, can nodulate *Phaseolus vulgaris*. Classification analysis of the 16S ribosomal sequence of OR191 have shown that strain OR191 is more closely related to *R. etli* and other bean symbionts than to *R. meliloti* (167). Unfortunately, this isolate was not further characterized with respect to the genes involved in nodulation. With the identification, cloning, and mutagenesis of the nodulation genes (see below), it has been shown that inactivation of a single gene drastically alters the host range of a particular strain (71, 118). *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii are very similar with respect to their symbiotic genes. However, their host ranges are very different. Exchange of one gene in the microsymbiont can switch the host range of the two biovars (246). Plant tests to determine the nodulation phenotype are nevertheless important, as they allow verification of predicted host range on the basis of characterization of the molecular structure of the signals that govern nodulation.

Infection and Nodulation Mechanisms

Root infection by rhizobia is a multistep process that is initiated by preinfection events in the rhizosphere. Rhizobia respond by positive chemotaxis to plant root exudates and move toward localized sites on the legume roots (11, 28, 56, 79, 98). Both *Bradyrhizobium* and *Rhizobium* spp. are attracted by amino acids, dicarboxylic acids present in the exudates, and very low concentrations of excreted components, such as flavonoids, that may not have high nutritional value (1, 4, 27, 129, 187). Apparently, chemotaxis is not required for nodulation, because flagellum-deficient mutants are still able to nodulate normally; instead, it has an influence on competition and the establishment in the rhizosphere (158, 163, 171).

Subsequently, the rhizobia attach to the plant root surface. For many rhizobia, primary target sites for infection are young growing root hairs, but there are no exclusive loci for rhizobial attachment (238). Initially it was speculated that attachment of *Rhizobium* and *Bradyrhizobium* spp. to legume roots was involved in host specificity. Specific adherence of compatible rhizobia was proposed to be mediated by specific binding of particular polysaccharide structures present on the bacterial cell surface to host plant lectins (19, 102). The lectin recognition hypothesis has been validated in one case in which clover plants transgenic for a pea lectin gene can be nodulated by *R. leguminosarum* bv. viciae, which normally does not nodulate clover (53a). Many studies failed to demonstrate any degree of host specificity at the attachment step (174, 193, 277). Smit et al. (233–237) undertook a detailed study on the attachment capacity of *R. leguminosarum* bv. viciae to pea root hairs under various physiological conditions. It was concluded that the conditions under which the rhizobia were grown strongly influenced the attachment to pea roots. Under specific growth conditions, lectin seems to be clearly involved in attachment of rhizobia (133). Smit et al. (234) demonstrated that rhizobial attachment is a two-step process, at least in the *Rhizobium*-pea system. In the first step, *R. leguminosarum* binds loosely as single cells to the root hair surface. In the second step, referred to as cap formation, additional bacteria accumulate at the adhesion site. However, it is still unclear which determinants are involved in each of these two steps. Under laboratory conditions, the firm attachment step is not even essential for nodulation (278). For *R. leguminosarum* bv. viciae, a Ca^{2+}-dependent adhesin, called rhicadhesin, mediates the initial direct attachment to pea root hair surfaces (234, 239). For cap formation, the firm attachment step, fibrillous appendages of (brady)rhizobia appear to be involved. These appendages can be cellulose fibrils (*R. leguminosarum*) or proteinous fimbriae (*Bradyrhizobium japonicum*) (234, 277, 279). However, it cannot be excluded that other bacterial macromolecules might be involved as well (110, 111).

At the surface of the root, and very probably also from a distance, rhizobia cause root hair branching, deforming, and curling (16, 269, 286). These phenomena are also observed with supernatants of induced rhizobia cultures. In all *Rhizobium*-plant interactions studied thus far, the active substances in the supernatant have been identified as lipooligosaccharides, also called Nod factors. These Nod factors are synthesized by means of some of the nodulation genes (150, 245, 263). In legumes, the region that is most susceptible to *Rhizobium* infection is just behind the apical meristem at the site of emerging root hairs (15). Elongated root hairs are sometimes infected, but this is rather unusual (29, 194). Mostly, the young root hairs can be curled sufficiently to entrap bacterial cells in a pocket of host cell wall. After entrapment, a local lesion of the root hair cell wall is formed by hydrolysis of the cell wall

(10, 179, 180, 265). The mechanism of hydrolysis of the cell wall is not known. Either the bacteria may induce hydrolytic enzymes that are responsible for localized cell wall dissolution, or the bacteria may exploit plant mechanisms such as those used when epidermal cells grow out into root hairs (132). Rhizobia enter the roots at the sites where root hair cell walls are hydrolyzed. The penetration occurs by invagination of the plasma membrane. The host plant reacts by depositing new cell wall material around the lesion in the form of an inwardly growing tube (29, 266). The tube is filled with proliferating bacteria surrounded by a matrix and becomes an infection thread. The infection thread grows toward the inner tangential wall of the root hair cell tip by a process of tip growth (10).

Concomitantly with formation of the infection thread, particular cortical cells divide to form a nodule primordium, and the infection thread grows toward these primordia (156, 181, 273, 284). In soybean (30, 69) and alfalfa (63, 284), cell division in the cortex can also occur prior to the invasion of the root hairs. In *Glycine max*, the first divisions occur in the hypodermis and spread to the outer layers of the cortex (30). Most division centers are not clearly associated with curled root hairs or (later) hairs with infection threads; most of these cell division centers are arrested and never form nodules (30). The root cortical cells through which infection threads will pass on their way to the nodule primordia change markedly before they are penetrated by an infection thread. Detailed cytological analyses have shown that microtubuli rearrange, that the nucleus migrates to the cell center, and that additional cell wall material is formed. On the basis of these cytological changes, it has been suggested that the cortical cells become prepared for infection thread penetration (267).

The location of the nodule primordia in the root cortex depends on the type of nodule formed by a particular plant (181). In general, in temperate legumes such as pea, vetch, and alfalfa, the primordium is formed from cells in the inner cortex (63, 156). These legumes form indeterminate cylindrical nodules and have a persistent apical meristem (180). This persistent activity of the meristem ensures nodule elongation, since new cells are constantly added to the distal end of the nodule (272). While the meristem is active, rhizobia are released from the infection threads into the plant cell cytoplasm (22, 108, 131, 181). The differentiation of micro- and macrosymbiont leads to the establishment of a central zone of the nodule, in which nitrogen is reduced (177, 272). Thus, in indeterminate nodules, nodule growth and functioning occur simultaneously, and all intermediates in differentiation can be observed in a single longitudinal section of a nodule. On the other hand, in most tropical legumes, such as soybean and French bean, nodules have a determinate growth pattern (179, 266). A nodule meristem is induced in the root outer cortex, and the bacteria are released into actively dividing meristematic cells, each daughter cell receiving rhizobia (179, 181). Meristematic activity is restricted to a short period. Following a round of successive divisions, the invaded meristematic cells differentiate simultaneously to form the nitrogen-fixing central tissue (181). As a result of this developmental pathway, nodule growth and function are dissociated. Determinate nodules do not elongate but enlarge, and only a single stage of plant and bacterial differentiation can be observed at any particular moment.

Besides the formation of infection threads through root hairs, which is most widely studied, the rhizobia may enter through cracks in the epidermis. In legumes such as *Arachis hypogaea* (peanut) and *Stylosanthes* spp., microsymbionts infect their hosts by "crack entry" (32, 33). In the presence of rhizobia, cell divisions are induced in the cortex of an emerging lateral root. Growth of the young root causes separation of cortical and epidermal cells and enables entry and intercellular spread of rhizobia. In both genera, no infection threads are formed, and rhizobia colonize the root apoplast presumably by cell wall digestion or, in *Stylosanthes* spp., by progressive collapse of outer root cells. Continuous host cell divisions result in development of a uniformly infected central tissue resembling the determinate nodule type (32, 33, 250).

Also, stem and root nodules of *Sesbania rostrata* are induced following crack entry by *A. caulinodans* at the base of dormant root primordia, which are present in rows along the length of the stem, or at the base of secondary roots in the case of root nodules (62, 65, 178, 264). Direct intercellular infection is followed by very active multiplication of the bacteria, forming wide intercellular spaces filled with azorhizobia. These spaces then extend inward as narrow, branched, intercellular infection threads which spread into the meristematic zone induced in the cortex. The subsequent release of bacteria into the cytoplasm of newly induced meristematic cells leads eventually to the development of a determinate nodule (65, 178, 263).

Bradyrhizobium sp. infects *Parasponia andersonii* (Ulmaceae), the only nonlegume plant genus to form nitrogen-fixing root nodules with rhizobia (261). Upon inoculation, the first sign of root nodule initiation is the formation of swollen multicellular root hairs. Simultaneously, the colonizing bacteria stimulate cell division in the outer cortex. In due course, these cell divisions cause development of callus-like bumps which rupture the epidermis, especially at the base of multicellular root hairs, and infection follows through these wounds (crack entry). After invasion, a large proliferation of intercellular rhizobia occurs, which is associated with damage to the host cells. Occasionally, intracellular rhizobia in single file surrounded by plant cell wall material are observed. Rhizobia are not endocytotically released from these infection threads, but the infection thread will change in nature as rhizobia differentiate into the nitrogen-fixing bacteroid form (147, 260, 261). This type of interaction is generally considered a primitive rhizobial infection, since the orderly invasion of the intercellular space (which leads to the formation of fixing threads), and the more damaging pathogenic type of interaction can occur in relatively close proximity (147). This type of invasion and infection thread formation has been described for root nodules of many genera in the family Caesalpinioideae and some in the family Papilionoideae, like *Andira* (46, 47).

A third mode of infection is observed in *Mimosa scabrella*, a tropical tree, where the rhizobial infection sites are at junctions of epidermal cells (44). The bacteria penetrate the radial walls and proliferate intercellularly. Occasionally, subepidermal root hairs were formed upon inoculation, but they were never infected. Penetration of outer root cells starts from irregular cell wall ingrowth and progresses in the cortical region through the primary wall layer, rather than by separating cells at the middle lamella. This usually results in elicitation of a plant host defense response. However, rhizobia can be released into the cells of the developing nodule meristem, and a normally indeterminate nodule is formed (44).

The route of infection is characteristic for the host, because the same bacteria can penetrate different host species by either crack entry, infection through intact epidermis cells, or root hair infection threads. A given legume is infected by the same type of mechanism regardless of the infecting strain (40, 206). Similarly, the structural and developmental characteristics of an efficient nodule are specified by the plant and not by the rhizobial strain, indicating that the host possesses the genetic information for symbiotic infection and nodulation and that the role of the bacteria is to switch on this plant developmental program (for a review, see reference 53).

Rhizobial Genes Controlling Infection, Nodulation, and Host Range: an Overview

The *Rhizobium* genes essential for infection and nodule formation can be divided into two classes. One class includes several sets of genes involved in the formation of the bacterial cell surface, such as genes determining the synthesis of exopolysaccharides (*exo* genes), lipopolysaccharides (*lps* genes), capsular polysaccharides or K antigens, and β-1,2-glucans (*ndv* genes) (21, 95, 148, 183, 203). Mutations in these genes disturb the infection process to various degrees, such as the inability to elicit the formation of infection threads, resulting in the formation of nonfixing empty nodules (Nod^+ Fix^- phenotype) (5, 149, 183, 201). A possible role of *exo* and *lps* genes in the determination of host specificity has been suggested, but no clear genetic evidence has yet been given that *Rhizobium* surface components are major determinants of host range specificity (64, 95, 183, 202).

The second class consists of the nodulation (*nod* or *nol*) genes. Inactivation of the nodulation genes can result in various in planta phenotypes, such as the absence of nodulation (Nod^-), a delayed but effective nodulation (Nod^d Fix^+), or changes in the host range. Some of the *nod* genes appear to be interchangeable for nodulation function between different species and biovars and are therefore designated as common *nod* genes (137). On the other hand, some *nod* genes are involved in the nodulation of a particular host and are hence called host-specific *nod* (*hsn*) genes (137). In most *Rhizobium* species studied to date, the *nod* genes reside on large symbiotic plasmids (pSym) that also carry the *nif* and *fix* nitrogen-fixing genes (166). In *Rhizobium loti* and *Bradyrhizobium* and *Azorhizobium* spp., the symbiosis-related genes are localized on the chromosome (3, 34, 85). Most *Rhizobium nod* genes are not expressed in cultured cells but are induced in the presence of the plant (for reviews, see references 75 and 136). This induction is caused mostly by flavonoids secreted by the plant (for reviews, see references 187 and 188) and also requires the participation of the transcriptional-activator protein NodD.

During the last few years, it has become clear that a major function of the *nod* genes is to ensure signal exchange between the two symbiotic partners (Fig. 1). In the first step, flavonoids excreted by the plant induce, in conjunction with the NodD protein, the transcription of bacterial *nod* genes (75, 221). In the second step, the bacterium, by means of the structural *nod* genes, produces lipooligosaccharide signals (Nod factors) (52a, 53, 240) that induce various root responses (245, 263). Mechanisms underlying host specificity depend on both the regulatory and the structural *nod* genes and will be discussed in further detail below.

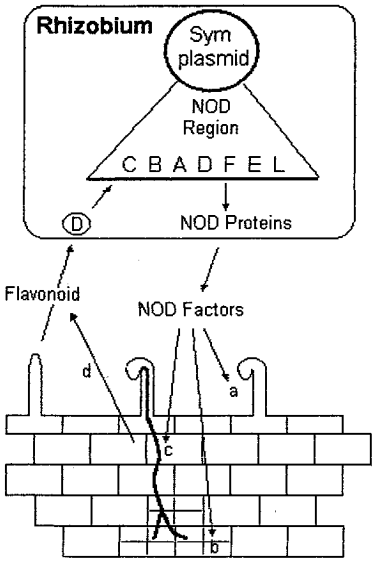


FIG. 1. Schematic representation of the interaction between *Rhizobium* species and legume roots. Plant-secreted flavonoids, in conjunction with the bacterial NodD protein, regulate the transcription of bacterial *nod* genes, here exemplified by the *nodABCDEFL* genes of *R. leguminosarum*. The bacterial *nod* gene products are involved in the synthesis of Nod factors. These Nod factors are able to induce the critical steps leading to nodule formation: (a) root hair deformation; (b) cortical cell division; (c) at least some steps of the infection process; and (d) increased flavonoid production. Reprinted with permission of the publisher from reference 78.

STRUCTURAL *nod* GENES AS HOST RANGE DETERMINANTS

Common *nod* Genes

The structural *nod* genes are classified into two groups, the common and host-specific *nod* genes (137). The common *nodABC* genes have been found in all *Azorhizobium*, *Rhizobium*, and *Bradyrhizobium* isolates studied so far (85, 166, 251). These genes have been called common *nod* genes because they are structurally conserved and functionally interchangeable between *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* species without altering the host range (166). In most species, the *nodABC* genes are part of a single operon (Fig. 2). However, in *R. etli*, *nodA* is separated by approximately 20 kb from the *nodBC* genes (274). Inactivation of the *nodABC* genes abolishes the ability to elicit any symbiotic reaction in the plant, including root hair curling (Hac^-), infection thread formation (Inf^-), cortical cell divisions, and nodule formation (Nod^-), regardless of the host, the mode of infection, the type of nodule development, and the nodule location (159, 166).

The *nodIJ* genes are present in *R. leguminosarum* bv. viciae and bv. trifolii, *B. japonicum*, *R. etli*, and *A. caulinodans* (70, 80, 90, 257, 275), and partial sequence data suggest that they are present in *R. meliloti* (123). They reside downstream of *nodC* and seem to be part of the same operon as *nodC*. Mutations in *nodIJ* result in a delay of nodulation in *R. leguminosarum* and *A. caulinodans* (70, 80) but have no detectable effect in *B. japonicum* (90). It has been proposed that NodI and NodJ proteins belong to a bacterial inner membrane transport system of small molecules (275).

Host-Specific *nod* Genes

Other nodulation genes have been identified that are not functionally or structurally conserved among rhizobia. These host-specific *nod* (*hsn*) genes are necessary for the nodulation of a particular host plant (137). In most cases, mutations cannot be fully complemented by the introduction of the corresponding genes from other rhizobia. Mostly mutations result in alteration or extension of the host range. *R. meliloti nodH* mutants result in a change of host range; they infect and nodulate vetch but fail to nodulate their normal host, alfalfa. The *nodQ* mutants, on the other hand, are able to infect both alfalfa and vetch (71, 118). In *R. leguminosarum* bv. viciae and bv. trifolii, the *nodE* product is the main factor that distinguishes the host range of nodulation. In *R. leguminosarum* bv. trifolii, in contrast to the wild type, *nodEF* mutants nodulate white and red clover poorly but have acquired the ability to infect and nodulate peas. When these *nodEF* mutants of *R. leguminosarum* bv. trifolii harbor the *nodE* gene of *R. legu-*

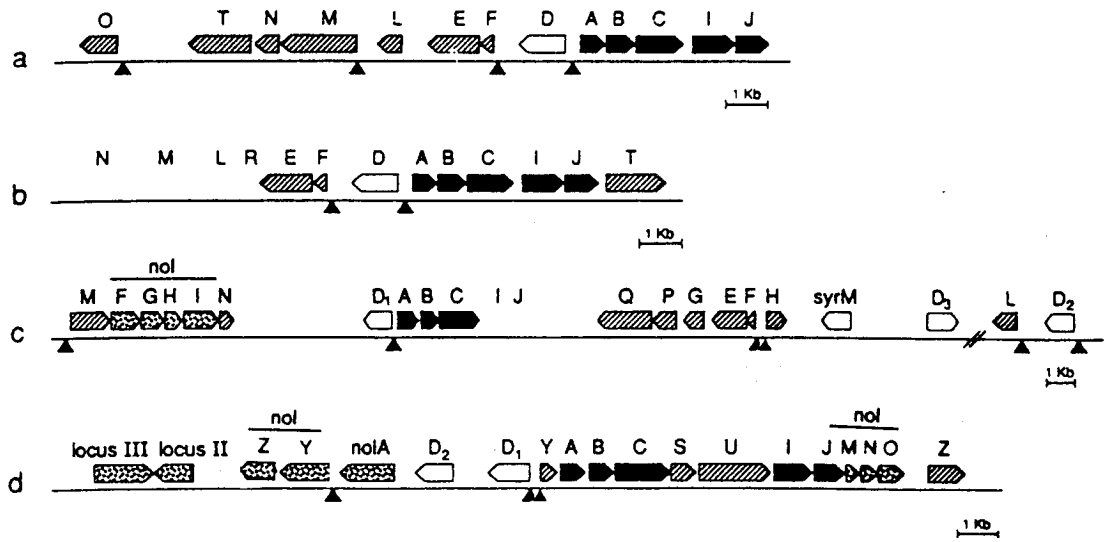


FIG. 2. Genetic organization of *nod* genes in *R. leguminosarum* bv. viciae (a), *R. leguminosarum* bv. trifolii (b), *R. meliloti* (c), and *B. japonicum* (d). The genes are presented as arrows which point in the direction of their transcription. Common *nod* genes are indicated by solid arrows, and host-specific *nod* genes are indicated by hatched arrows. The *nodD* genes are indicated by white arrows. *nol* genes, unknown open reading frames, and other *nod* loci are indicated by stippled arrows. The *nodX* gene of *R. leguminosarum* bv. viciae TOM is not indicated but is located downstream from *nodJ* and presumably in the same operon (41). Black triangles indicate the position of the *nod* boxes. Updated from reference 217.

minosarum bv. viciae, they have an extended host range to *Vicia* and *Lathyrus* species (246). In *Rhizobium* sp. strain NGR234, mutation of *nodS* causes a Nod$^-$ phenotype on *Leucaena leucocephala* but gives a normal phenotype on *Macroptilium atropurpureum* (152). In a similar way, the *nodS* mutations in *R. tropici* CIAT899 and *A. caulinodans* cause a loss of nodulation of *L. leucocephala* (280). Waelkens et al. (280) also demonstrated that the *nodS* mutation of *Rhizobium* sp. strain NGR234, *R. tropici* CIAT899, and *A. caulinodans* exhibits a Nod$^-$ phenotype on *Phaseolus vulgaris*. The *nodSU* genes have also been identified in *B. japonicum* and *R. fredii*, but no phenotype has been described for these genes (90, 143). Krishnan et al. (143) postulated that poor expression of the *R. fredii nodS* gene is the cause of the lack of *Leucaena* nodulation. Several loci involved in host-specific nodulation in *B. japonicum* have been identified. *nodVW* mutants of *B. japonicum* have lost the ability to nodulate mung bean (*Vigna radiata*), cowpea (*V. unguiculata*), and siratro (*Macroptilium atropurpureum*), but the nodulation of soybean (*Glycine max*) is only marginally delayed (89). The *nodZ* gene of *B. japonicum* exhibits a host-specific characteristic at both species and cultivar levels, since *nodZ* mutants fail to nodulate siratro and shows an altered nodulation ability on a few varieties of soybean (182, 252).

Within the classic cross-inoculation groups of the *Rhizobium*-legume symbiosis, examples have been seen of more precise host-specific interaction at the plant cultivar level. Bacterial genes appear to control this cultivar specificity by acting as positive or negative traits. The first report on such a positive-acting gene was for the *R. leguminosarum* bv. viciae-pea symbiosis, in which a single gene, *nodX* from strain TOM, was identified as being responsible for overcoming the nodulation resistance of the cultivar Afghanistan peas (41). A similar case is the *nolA* gene identified in *B. japonicum* USDA110. The presence of *nolA* allows serocluster 123 isolates to nodulate soybean plants having USDA123-restricting genotypes. The nodules formed, however, were ineffective for symbiotic nitrogen fixation (211).

An example of a nodulation gene that acts negatively on the nodulation ability of a certain cultivar is *nodM*, present in *R. leguminosarum* bv. trifolii TA1. The ability of strain TA1 to nodulate the subterranean clover cultivar Woogenellup is cold sensitive; at 28°C strain TA1 forms an effective symbiosis with this cultivar, but at 22°C little or no sign of nodulation is apparent (84). However, inactivation of *nodM* restores normal nodulation of cv. Woogenellup by strain TA1 (154). On the other hand, the introduction of *nodM* from the related *R. leguminosarum* bv. trifolii ANU843, which is able to fully nodulate cv. Woogenellup, again suppresses the nodulation of cv. Woogenellup by TA1, which indicates that the *nodM* genes of the two strains are interchangeable (154). In addition, the *nodT* gene from *R. leguminosarum* bv. trifolii ANU843 can act as a dominant suppressor of the negatively acting *nodM* and allows *R. leguminosarum* bv. trifolii TA1, which lacks a *nodT* gene, to nodulate cv. Woogenellup (155). In strain ANU843, however, there is no discernible phenotype for NodT recognized in nodulation of cv. Woogenellup (257).

R. fredii USDA257, a soybean symbiont, is able to form nodules on primitive soybean varieties, such as Peking, but fails to nodulate several agronomically advanced varieties, like McCall (107, 130). Recently, several negative-acting genes present in strain USDA257 have been identified. For instance, a Tn*5* insertion in *nolC*, a chromosomal locus, enables strain USDA257 to form Fix$^-$ nodules on soybean cv. McCall, but it also exhibits abnormalities on cv. Peking and expresses other pleiotropic effects (145). In contrast, the second group of Tn*5* mutants, inactivating one of the genes *nolB*, *nolT*, *nolU*, *nolV*, *nolW* or *nolX*, all located on the pSym plasmid, enables *R. fredii* USDA257 to form fully fixing nodules on cv. McCall (170). Recently, Krishnan and Pueppke (146) discovered that *R. fredii* USDA257 is able to form nodules on *Erythrina costaricensis* but not on six other *Erythrina* species. Inactivation of *nolBU* or *nolC* broadens the symbiosis to include other *Erythrina* species (146).

In several cases, *nod* genes have been identified in mutants that exhibit no apparent defects in nodulation. For example, mutations of *nodT* in *R. leguminosarum* bv. viciae and bv. trifolii ANU843 (257) and of *nodSU*, *nodY*, and *nolYZ* in *B. japonicum* (90, 93) are not associated with an apparent altered

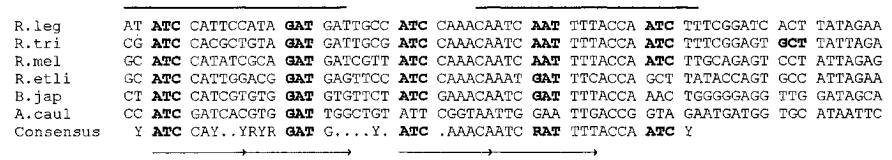


FIG. 3. Sequence comparison of six *nod* boxes upstream from the common *nod* gene operons in *R. leguminosarum* bv. viciae (R.leg) (231), *R. leguminosarum* bv. trifolii (R.tri) (224), *R. meliloti* (R.mel) (209), *R. etli* (274), *B. japonicum* (B.jap) (182), and *A. caulinodans* (A.caul) (88). The consensus sequence was defined by Spaink et al. (243). The $ATCN_9GAT$ repeats, or related motifs, are indicated in boldface type. Y, pyrimidine; R, purine; ●, arbitrary base. The pairs of tandem arrows indicate the 9-bp repeats in the alternative *nod* box proposed by Wang and Stacey (282). The solid lines indicate the segments shown by interference footprinting to be critical for DNA binding according to Fisher and Long (76).

nodulation phenotype. These results are unexplained, but it is possible that the plants used in the assay are relatively insensitive to certain types of symbiotic effects. Alternatively, it is possible that the mutations are critical for the nodulation of some cultivars of plants not yet used in the assays.

REGULATORY *nod* GENES AS HOST RANGE DETERMINANTS

nodD Gene

The expression of the *Rhizobium* structural *nod* genes requires the presence of a plant signal, generally flavonoids, and the presence of the regulatory protein NodD. The NodD protein binds to conserved DNA sequences upstream of the inducible *nod* operons, called *nod* boxes. In the presence of plant signals, the NodD protein acts as a transcriptional activator (74, 209). This NodD regulatory system is present in all *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* strains studied so far. However, there are variations between species in the number of *nodD* copies present, and as many as five copies have been observed in some species (87, 159, 271a).

NodD as a member of the LysR family of transcriptional activators. The different *nodD* genes are conserved at the nucleotide sequence level, and the corresponding proteins have been classified as members of the LysR family of transcriptional activators (106). This group of regulator proteins shares several common features, as described by Schlaman et al. (222). These proteins all require an inducing compound for activation and all possess a putative helix-turn-helix motif in the amino region, characteristic for the DNA-binding ability. The NodD protein in *R. leguminosarum* bv. viciae and *R. meliloti* has been found to bind to the *nod* box, even in the absence of an inducer (74, 113). However, in *R. meliloti* AK41 and *Azorhizobium* species binding of NodD to the *nod* promoter is enhanced in the presence of its inducer (88, 139). The *nod* box was originally defined in *R. meliloti* as a 47-bp consensus sequence required for *nod* gene induction (209). In the genetically distant *B. japonicum* and *A. caulinodans*, less highly conserved *nod* boxes have been identified and shorter consensus sequences have been proposed (88, 282). A comparison of various divergent *nod* boxes revealed the presence of two inverted repeats $ATCN_9GAT$ (Fig. 3), which have a repeat structural feature typical for DNA targets that are symmetrically bound by protein dimers or tetramers (184). The presence in all *nod* boxes of such structures favors the hypothesis that NodD binds to the *nod* box as multimers (88). This model, however, is in disagreement with the model proposed by Fisher and Long (76). They provide strong arguments, based on interference footprints and DNA-phasing analysis, for two separable binding sites for NodD on one face of the DNA helix, in a region corresponding to the original consensus *nod* box (Fig. 3). This region does not contain completely the two paired repeats $ATCN_9GAT$, which lie on opposite faces of the DNA helix, as indicated in Fig. 3.

The specificity of each different NodD occurs at the level of amino acid sequence. Several hybrid NodD proteins have been constructed and exhibit the flavonoid specificity of the NodD product constituting their C-terminal end (116, 248). This indicates that the signal specificity is located primarily at the C-terminal part of the protein, which is less highly conserved than the N-terminal part. In addition, the carboxyl part of the protein exhibits some resemblance to animal steroid receptors, which are also known to interact with some flavonoid ligands (100). However, some of the mutations that modify the flavonoid specificity map in the N-terminal region (169, 248). Thus, the flavonoid specificity depends on the overall tertiary structure of NodD protein.

NodD as a membrane protein. In *R. leguminosarum* bv. viciae, the NodD protein is localized in the bacterial cytoplasmic membrane, presumably inserted only in the cytoplasmic monolayer (222). In *R. meliloti*, the NodD protein has been localized in the cytosol and migrates toward the cytoplasmic membrane when appropriate flavonoids are added (139). Also, flavonoids have been reported to accumulate in the cytoplasmic membrane (119, 196). Recently, Hubac et al. (119) have shown that the absorption of luteolin by *R. meliloti* in the inner and outer membranes involves the NodD proteins. These observations support the suggestion that the site of interaction between NodD and the flavonoid is likely to occur in the inner membrane (221). So far, there is no direct evidence that flavonoid inducers bind directly to NodD proteins. However, genetic evidence is in line with this hypothesis: (i) point mutations in *nodD* may change the sensitivity to additional inducers (25, 26, 169, 248); and (ii) the transfer of a *nodD* gene to a foreign *Rhizobium* strain is linked to the transfer of sensitivity to a given set of flavonoids (116, 247). Since flavonoids are required for activation of the NodD protein, they presumably induce a conformational change in the protein. This notion is supported by the fact that it is possible to construct mutants and hybrid *nodD* alleles which can activate transcription in the absence of flavonoids (25, 244).

In *R. leguminosarum* bv. viciae and *R. meliloti*, the soluble form of NodD binds to *nod* box sequences even in the absence of inducers, although in *R. leguminosarum* bv. viciae a minor fraction of cytoplasmic membrane-located NodD is able to bind to the *nod* box as well (74, 217). Using migration retardation essay with the flavonoid-independent *nodD3* allele of *R. meliloti* Fisher and Long (76) have shown that NodD3 induces or stabilizes a bend in the *nod* box upon binding and that the bend center lies between the two NodD-binding sites. It is possible that the specific bend induced by NodD3 is immediately present in an "active" mode and that this protein bends the *nod* box more or less than the flavonoid-dependent NodD proteins do (76). In *R. meliloti*, a chaperone-like protein showing homology to GroEL of *Escherichia coli* is necessary for the

TABLE 2. Effects of flavonoids, in conjunction with various NodD proteins, on *nod* gene expression[a]

Compound	Substitution position							Activity of NodD protein[b]							
	3	4	5	7	3′	4′	5′	RtrD1	Rt	Rl	RmD1	RmD2	NGR234	Bj	RpD2
Flavones															
Luteolin			OH	OH	OH	OH		+	++	++	++	−	++	−	+
Apigenin			OH	OH	OH			++	++	++	+	−	++	+	++
—[c]				OH	OH	OH		ND	ND	++	++	−	ND	ND	ND
Chrysoeriol			OH	OH	OCH_3	OH		ND	ND	ND	++	−	ND	ND	+
—					OH	OH		ND	ND	ND	−	−	ND	ND	ND
—				OH		OH		++	++	ND	+	ND	++	+	ND
Chrysin			OH	OH				++	+	−	−	−	++	−	+
Galangin	OH		OH	OH				−	−	−	ND	ND	ND	ND	+
Flavonols															
Myricetin	OH		OH	OH	OH	OH	OH	−	−	−	−	−	+	ND	ND
Quercetin	OH		OH	OH	OH	OH		−	−	−	−	−	++	−	ND
Kaempferol	OH		OH	OH		OH		−	−	−	−	−	++	+	+
Flavanones															
Eriodictyol			OH	OH	OH	OH		−	+	++	+	−	ND	−	+
Naringenin			OH	OH		OH		++	++	++	−	−	++	−	++
Hesperitin			OH	OH	OH	OCH_3		−	−	++	−	−	++	ND	+
Isoflavones															
Genistein			OH	OH		OH		−	−	−	−	−	++	++	++
Daidzein				OH		OH		ND	−	−	−	−	++	++	ND
Chalcone															
4,4′-Dihydroxy-2′-methoxychalcone		OH				OH		ND	ND	ND	++	++	ND	ND	ND

[a] Data from references 43, 53, and 270.

[b] The *nod* inducing activities of various flavonoids were recorded for rhizobial strains harboring the *nodD* genes of *R. tropici* NodD1 (RtrD1); *R. leguminosarum* bv. trifolii (Rt), *R. leguminosarum* bv. viciae (Rl); *R. meliloti* NodD1 (RmD1) and NodD2 (RmD2); *B. japonicum* (Bj) and *R. leguminosarum* bv. phaseoli (Rp). The different activities are related to the maximum inducing activity recorded for these strains. ++, 50 to 100%; +, 10 to 50%; −, 0 to 10% of the maximum induction. ND, not detected.

[c] —, compounds for which no commercial name is available yet.

transcriptional activity of NodD3 (160). It is conceivable that this protein is necessary for the translocation of NodD from the cytoplasmic membrane to keep it in a proper, soluble conformation (221).

Plant signals activating NodD. The activation of *nod* expression by NodD proteins requires, with some exceptions, the presence of compounds in plant exudates (122, 175, 208). They were first identified as flavonoids or related compounds derived from plant phenylpropanoid metabolism, which is also known to provide molecules involved in plant defense (73, 185, 197). Flavonoids are released as aglycones or glycosidic conjugates. The latter are less active but have a higher solubility in water, and they can be converted to active forms by bacterial glycosidases (104, 120, 168). Recently, Phillips et al. (189) identified nonflavonoids, namely, the two betaines trigonelline and stachydrine, as being two major compounds from alfalfa seed exudates activating the NodD2 protein in *R. meliloti*. Betaines are chemically quite different from flavonoids, have different physicochemical properties (for example, water solubility) and are synthesized by different metabolic pathways in the host plants. It is interesting that these two types of molecules (flavonoids and betaines), which are chemically so different, are able to cause transcriptional activation of the isoforms of the same protein.

The nature and amounts of the compounds exuded depend on the plant and its stage of development. For alfalfa, soybean, and bean, the spectrum of flavonoids present in seed exudates is different from that present in root exudates (94, 103, 120, 121). Inoculation with an infective rhizobial symbiont causes a change in the internal flavonoid pool of the root. This second wave of flavonoids leads to an increased *nod* gene-inducing activity, by as much as 10-fold, as detected for white clover (207), vetch (268), soybean (223), bean (39), *Lotus* species (35), and alfalfa (38). In several cases, flavonoids without inducing properties have been shown to inhibit *nod* gene activation by effective inducers (54, 73, 101, 141, 186). The anti-inducers usually have similar structures to those of the inducers, and inhibition can be overcome by increasing the concentration of the inducers (186). Also, synergistic interactions have been observed and can be explained if one considers that NodD acts as a multimer (103, 105).

NodD as a determinant of host range. It is apparent that the spectrum of flavonoid specificity of the endogenous NodD protein correlates with the broadness of the host range. NodD proteins from narrow-host-range rhizobia, like *R. meliloti*, *R. leguminosarum* bv. viciae, and *R. leguminosarum* bv. trifolii, respond to few flavonoids, while NodD from the broad-host-range *Rhizobium* NGR234 has a larger spectrum of inducing compounds, including even the monocyclic aromatic compounds vanillin and isovanillin (101, 151) (Table 2).

Several lines of genetic evidence have established that *nodD* genes, because of the flavonoid specificity, are determinants of host specificity. (i) Some mutations in *nodD* cannot be complemented by a *nodD* from other *Rhizobium* species (116, 247). For example, the *R. meliloti nodD1* gene cannot complement the *nodD* mutation of NGR234 for nodulation of siratro. This lack of complementation is due to the inability of *R. meliloti nodD1* to respond to the signals exuded by siratro. (ii) Transfer of the *nodD* gene alters the host range. The transfer of the *nodD1* gene from *Rhizobium* sp. strain NGR234 into *R. meliloti* results in the transfer of the ability to nodulate siratro (116). (iii) Some point mutations in the *nodD* gene of *R. trifolii* can extend the host range to the nonlegume *Parasponia* species (169). (iv) A *nodD* hybrid gene, constructed in vitro from *R. meliloti* and *R. trifolii nodD* genes, extends the host range to include tropical legumes (244). These experiments indicate

that different NodD proteins have different inducer specificities and, as a consequence, influence the host specificity of nodulation (116, 247).

NodD regulation. The *nodD* gene exists as a single gene in *R. leguminosarum* bv. viciae and bv. trifolii (59, 122) and probably also in *Azorhizobium* species (87). Inactivation of the *nodD* gene consequently confers a Nod$^-$ phenotype. Other rhizobia possess multiple copies of the *nodD* gene: *R. fredii* USDA 191, *Rhizobium* sp. strain NGR234, and *B. japonicum* harbor two copies, of which NodD1 is the activator of *nod* genes whereas no such a function could be assigned to NodD2 (3, 23, 91). *R. meliloti* and *R. leguminosarum* bv. phaseoli possess three *nodD* genes (42, 92, 115). Mutation in one of the three *nodD* homologs of *R. meliloti* reduces nodulation in a host-dependent manner (99, 114). Up to five copies of the *nodD* gene have been identified in *R. tropici* CIAT899 (271a).

The significance, in terms of control of specificity, of these *nodD* reiterations is not clear, because there is no correlation between the number of *nodD* genes and the broadness of the host range: the narrow-host-range *R. leguminosarum* bv. phaseoli has three copies (42), whereas the broad-host-range *Rhizobium* sp. strain NGR234 possesses two *nodD* copies, of which only one seems to be involved in *nod* gene induction (23). It is possible that NodD plays other roles besides induction of *nod* genes. This can be explored only with strains having multiple *nodD* copies by analysis of mutants at various levels of bacterium-plant interactions.

The various *nodD* genes also differ in their regulation. The single *nodD* gene negatively regulates its own transcription in *R. leguminosarum* bv. trifolii and bv. viciae (208, 243). In *B. japonicum*, *R. leguminosarum* bv. phaseoli, and *R. fredii*, the *nodD1* gene is preceded by a *nod* box sequence (3, 43, 282). For the first two species, the *nodD1* transcription level is enhanced in the presence of NodD1 protein and certain flavonoids, independently of other *nod* genes (3, 43).

In *R. meliloti*, the expression of *nodD3* and *syrM*, another member of the LysR family, is strongly intertwined in a complex way: the *syrM* gene product activates the expression of *nodD3*, which in turn activates the expression of *syrM* (138, 210, 259). The two genes constitute a self-amplifying positive regulatory circuit. Whereas NodD1 and NodD2 are specifically activated by plant compounds, plant inducers have little effect on the *syrM-nodD3* interaction. In the free-living state, *R. meliloti* strains with *syrM* and *nodD3* present in a single copy show no detectable expression of either gene (259). However, when both are carried on a multicopy plasmid, SyrM and NodD3 are present at sufficiently high concentrations to induce high levels of *nodABC* expression in the absence of an inducer (176, 259). This suggests that one or both genes might be repressed in the free-living state (259). To a lesser extent, the *nodD2* gene influences *syrM* expression (138) and *nodD1* influences *nodD3* expression (162). Interestingly, SyrM also regulates the synthesis of the *exo* genes, indicating that SyrM could coordinately regulate the metabolism of exopolysaccharide and of the Nod factors, both of which are involved in the infection process (12, 210). Recently, *syrM* has also been identified in *Rhizobium* sp. strain NGR234 and *R. leguminosarum* bv. phaseoli (173, 198).

Another role of NodD seems to be the regulation of *nod* genes as a function of the concentration of combined nitrogen. *R. meliloti* NodD3 and *B. japonicum* NodD1 are involved in the repression of the *nod* regulon in the presence of an excess of combined nitrogen (66, 281). In *R. meliloti* two NtrC-binding sites were found upstream of *nodD3* (135), whereas in *B. japonicum* neither NifA nor NtrC appears to be involved.

A repressor of *nod* gene expression, NolR, has been identified in several strains of *R. meliloti*, but no NolR function could be found in the well-studied strain *R. meliloti* 1021. NolR binds to the promoter region of *nodD1* and *nodD2* and not to any of the inducible *nod* promoters. Upon binding, it controls negatively the expression of these two regulatory genes and consequently of the *nod* regulon (139, 140). A mutation in the repressor results in a delayed nodulation phenotype. Thus, in some strains, *nolR* controls the fine-tuning of *nod* gene expression (139). In *R. meliloti* 1021, the lack of NolR function is shown to be due to a single insertional mutation in the C-terminal coding sequence which abolishes the DNA-binding activity (37). Sanjuan et al. (213) isolated, by hybridization to the *R. meliloti nolR* locus, a corresponding repressor locus in *B. japonicum*. Mutations in this loci result in an elevated level of *nod* gene induction. Hybridization studies suggest that *nolR* does not exist in *R. leguminosarum* bv. viciae. It was shown, however, that the chromosomal background in *R. leguminosarum* strongly influences the expression of *nod* genes, indicating that an additional regulator may act in some *R. leguminosarum* strains as well (220). In *A. caulinodans*, at least three other proteins, smaller than NodD, were found to bind to *nod* box DNA, but their function is unknown (88).

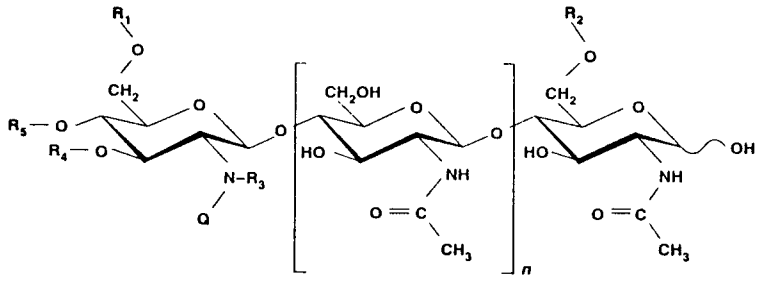
The inducible *nod* genes are not transcribed in the bacteroids, and thus their expression stops after the bacteria are released from the infection thread (218, 230). Apparently, in *R. leguminosarum* bv. viciae this is caused by ineffective binding of NodD to the *nod* box, because of either a conformational change of the protein or its presence in another complex (219). In the bacteroids of *R. leguminosarum* bv. viciae, the level of *nodD* expression is approximately 35% of that of the free-living cells, and this may be caused by a bacteroid-specific repressor protein (219). In *R. meliloti*, the *nodD3* gene is expressed in the meristematic region and the infection zone of the nodule, while *syrM* is expressed in the central tissue of the nodule (230). Swanson et al. (259) showed by in situ expression studies the interdependence of *nodD3* and *syrM* expression in the nodule: no *nodD3* expression occurred if *syrM* was mutated, and, conversely, no *syrM* expression was detected if *nodD3* was mutated.

Other *nod* Regulatory Genes

Other *nodD* regulatory genes, which are determinants of host specificity, have been found in *Bradyrhizobium* species. Mutants with mutations of *nodVW* have lost the ability to nodulate cowpea, mung bean, and siratro, but nodulation on soybean is only marginally delayed (89). The predicted amino acid sequence of NodVW suggests that these gene products are members of the family of two-component regulatory systems. This led to the hypothesis that NodV responds to an environmental stimulus and that after signal transduction, NodW may be required to positively regulate the transcription of one of several unknown genes involved in the nodulation process (89). Recently, Sanjuan et al. (214) found that *nodW*, in conjunction with *nodD1*, is essential for maximal induction of the common *nod* genes and *nodD1*. How the NodW protein is involved in the regulation is not known. The possibility that other, as yet unknown, genes are under control of NodW has not been ruled out. Also, the possibility that NodW is involved in modifying plant signals required for NodD activation of *nod* promoters should be considered (214). Grob et al. (96) reported the identification of a *B. japonicum* gene, *nwsB*, a *nodW* suppressor, which when overexpressed from a strong promoter is able to suppress the Nod$^-$ phenotype from the *nodW* mutant. The *nwsB* gene is preceded by a long open reading frame, *nwsA*. The protein products of the *nwsAB* gene pair also appear to be two-component regulators and are most similar to

TABLE 3. Structures of lipooligosaccharides produced by different *Rhizobium* strains[a]

Strain	*n*	Q	R_1	R_2	R_3	$R_{4,5}$	Reference
R. leguminosarum bv viciae RBL5560	2, 3	$C_{18\Delta2\ 4\ 6\ 11}$ $C_{18\Delta11}$	CH_3CO	H	H	H	245
R. meliloti AK41	1, 2, 3	$C_{16\Delta2\ 9}$ $C_{16\Delta2\ 4\ 9}$	H CH_3CO	SO_3H	H	H	226
R. meliloti 2011	2, 3	$C_{16\Delta9}$ $C_{16\Delta2\ 9}$ $C_{16\Delta2\ 4\ 9}$ (Ω-1)-OH C_{18-26}	H CH_3CO	SO_3H	H	H	51, 52, 150
R. tropici CFN299	3	$C_{18\Delta11}$	H	SO_3H	CH_3	H	191
B. japonicum USDA110	3	$C_{18\Delta9}$	H	2-*O*-Me-Fuc	H	H	212
B. japonicum USDA135	3	$C_{18\Delta9}$ C_{16}	H CH_3CO	2-*O*-Me-Fuc	H	H	31
B. japonicum USDA61	2, 3	$C_{18\Delta9}$	H CH_3CO	Fuc 2-*O*-Me-Fuc	H CH_3	H	31
R. fredii USDA257	1, 2, 3	$C_{18\Delta11}$	H	Fuc 2-*O*-Me-Fuc	H	H	14
Rhizobium sp. strain NGR234	3	$C_{18\Delta11}$ C_{16}	H	2-*O*-Me-Fuc 2-*O*-Me, 4-*O*-SO_3H-Fuc 2-*O*-Me, 3-*O*-CO-CH_3-Fuc		H NH_2CO	192
A. caulinodans ORS571	2, 3	$C_{18\Delta11}$ C_{18}	H NH_2CO	H D-Arabinosyl	CH_3	H	172



[a] The chitin oligomer and the acyl moiety (Q) are present in all Nod metabolites. The number (*n*) of *N*-acetylglucosamine residues can vary. Q can vary in length and in the number of unsaturated bonds. Several different substitutions to the sugar backbone occur (R_1 to R_5), which are listed in the table. Me-Fuc, methylfucose.

the protein products of the *B. japonicum* gene pair *nodVW* (97). Cross talk occurs between NwsAB and NodVW, since the activity of NwsB depends on either the NodV or the NwsA sensor kinase. The physiological importance of cross talk between NodVW and NwsAB in *B. japonicum* remains unclear (97).

Another gene of *B. japonicum*, *nolA*, plays a role in the nodulation of soybean genotypes that are restricted in nodulation by members of *B. japonicum* serocluster 123 (211). The N terminus of the predicted gene product of *nolA* has strong similarity with the N terminus of MerR, the regulator of mercury resistance genes. Although no *nod* box sequence could be found upstream of *nolA*, the expression of *nolA* is moderately induced by soybean seed extracts and the isoflavone genistein (211). The *nodZ* gene of *B. japonicum* exhibits a host-specific function for the nodulation of siratro and a few varieties of soybean (182, 252). Functional analysis showed that *nodZ* is involved in the production of the Nod factor but is not regulated by NodD. Surprisingly, it shows an elevated expression in the bacteroids, although NifA is not required for *nodZ* expression and expression is not influenced by oxygen concentration (252).

Several *nod* genes of *R. fredii* are not preceded by a *nod* box (20, 144, 170). *nolC*, a chromosomal locus of *R. fredii* USDA257, was first identified as a negative regulator of cultivar-specific nodulation of soybean cv. McCall (144). However, further investigation revealed that *nolC* is also detrimental to nodulation of other soybean plants, like cv. Peking, has other pleiotropic effects (145), and acts as a negative regulator for specific nodulation of several *Erythrina* species (146). *nolC* has strong sequence homology to *dnaJ*, which encodes a heat shock protein in *Escherichia coli* and contains a regulation function (144). *nolC* is constitutively expressed, and in planta histological studies confirm that its expression is not temporally regulated but, rather, is sustained throughout nodule development, including the preinfection stage (145). A second gene cluster, consisting of *nolBTUV*, *nolW*, and *nolX*, which is present on the pSym plasmid, is involved in the cultivar-specific nodulation of soybean cv. McCall (170). Insertion mutations of each gene extend the host range to soybean cv. McCall without exerting any effect on other host plants. Also, the inactivation of *nolBU* broadens the host range of *R. fredii* USDA257 to include several new *Erythrina* species (146). Expression of *nolBTU* and *nolX* is induced as much as 30-fold by flavonoid signal molecules, even though these genes lack *nod* box promoters. In planta analysis verified that these genes are expressed continuously from preinfection to the stage of the functional nodule (170). Another *nod* box-independent *nod* gene has been identified in *R. fredii* USDA201, namely, *nolJ*, which requires a functional *nodD1* gene for induction (20). *nolJ* shares no sequence homology with any of the previously reported common or *hsn* nodulation genes.

Nod FACTORS

Rhizobia produce and secrete soluble factors in response to the inducing flavonoids from the plant. The *nod* genes are essential for this process (268, 269). Recently, the chemical structures of these nodulation factors, also called Nod factors, have been determined (14, 31, 150, 172, 191, 192, 212, 226, 245). Uniformly, they consist of an oligosaccharide backbone of β-1,4-linked *N*-acetyl-D-glucosamine varying in length from three to five sugar units. A structurally varied fatty acid group is attached to the nitrogen group of the nonreducing amino sugar moiety. The presence of other substitutions is dependent on the species and strain (Table 3).

Biological Activities of Nod Metabolites

Nod factors are considered to be the main *Rhizobium* nodulation signal molecules, since the purified molecules are able to induce many of the plant responses observed in early stages of symbiosis (245, 263). Lipooligosaccharides from several species of rhizobia are able to elicit root hair deformation and induce nodule primordia in a host-specific way, indistinguishable from the nodule meristem in the first stage of normal nodule organogenesis (105a, 150, 172, 200, 212, 245, 263). Normally, the formation of shepherd's crooks has not been detected by treatment with Nod factors. Only in the case of the very promiscuous *Macroptilium atropurpureum* does the application of Nod factor provoke marked shepherd's crook-type curling of the root hair. This even occurs with Nod factors of *Rhizobium* strains which are not able to nodulate *M. atropurpureum*, although a higher concentration of Nod factor is needed (200). In *Medicago sativa* and *Glycine soja*, the nodule meristems are capable of developing further into full-grown nodules which have anatomical and histological features of genuine nodules induced by *Rhizobium meliloti* and *Bradyrhizobium japonicum* (254, 263). Recently, nodule-like structures following treatment with Nod factors have been observed with soybean, siratro, bean, and *Sesbania* species (165, 172, 200). Purified Nod factors are not able to promote the formation of genuine infection threads, but certain effects of the lipooligosaccharides have been observed which suggest that they are involved in the infection process. The formation of pre-infection thread structures is observed after treatment of *Vicia* roots with the lipooligosaccharides of *R. leguminosarum* bv. viciae (267). Another indication comes from the observation that the transcription of some early nodulins, which are specifically expressed in the infection thread, is also induced with isolated Nod metabolites (117, 128, 177, 215).

The lipooligosaccharides of *R. leguminosarum* bv. viciae are able to elicit the production and secretion of additional flavonoids in the roots of *Vicia sativa* (195). These additional flavonoids are efficient inducers of the NodD-dependent *nod* gene expression and are also found by treatment of plants with infective rhizobia (39, 268). In a similar case, the response of soybean roots to either *B. japonicum* inoculation or treatment with Nod factors of *B. japonicum* or *Rhizobium* sp. strain NGR234 resulted in the accumulation of flavonoid molecules in soybean root exudates (223).

It has been shown that application of cytokinin and auxin transport inhibitors induces the formation of pseudonodules similar to the formation induced by treatment of Nod factors (36, 109, 200). Since Nod factors share properties with plant hormones, they may be regarded as "hormone-like" molecules. However, Nod factors possess another essential characteristic, namely, the ability to curl root hairs. These two properties of Nod factors allowed the *nodABC* deletion mutant of *Rhizobium* sp. strain NGR234 and of *B. japonicum* to enter the roots of *Macroptilium atropupureum* and *Glycine max*, respectively, in the presence of NGR234 Nod factor (199, 200). The Nod factor of NGR234 also allowed *R. fredii* USDA257 to enter and fix nitrogen on the nonhost *Calopogonium caeruleum* (199).

The activity of Nod factors can also be determined by the presence of plant enzymes involved in the metabolism of the Nod factor. Staehelin et al. (253) have shown that the Nod factors of *R. meliloti* are rapidly inactivated in the rhizosphere of alfalfa by the action of chitinases and that the rate of degradation depends on the structural modification of the Nod factor. The presence of the sulfate group on the O-6 position of the reducing end of the *R. meliloti* Nod factor strongly protects against degradation by purified plant chitinases and intact plant roots (253). The finding that the Nod factors are substrates for plant chitinases and the fact that the Nod factors are cleaved by chitinases at a different rate might be an important determinant in the host specificity (225).

Biochemical Function of the *nod* Gene Products

Knowledge of the biochemical function of several Nod gene products involved in the biosynthesis has been obtained in different ways: (i) comparison of the Nod factors of wild-type and mutant strains, (ii) prediction of their function by searches for structural homology with already known proteins, and (iii) physiological or biochemical study of the *nod* genes products. The information obtained by comparison of the deduced amino acid sequences with other proteins of known function is summarized in Table 4.

Common *nod* genes and the molecular backbone. It has been shown recently that the common *nodABC* genes are sufficient to synthesize the backbone of signal molecules consisting of N-acylated glucosamine oligosaccharides (245). Results obtained by Geremia et al. (83) from in vitro labelling studies with cell extracts of several *Azorhizobium* mutants and a construct that produces the NodC protein in *E. coli*, strongly suggest that NodC functions as an *N*-acetylglucosaminyltransferase involved in the synthesis of the chitin backbone. This conclusion is consistent with the results of homology studies, which indicated that positions of NodC are homologous with a domain of various chitin synthases and cellulose synthases (6, 24, 48). It is not yet clear how the exact number of monomers in the oligosaccharide backbone (tri-, tetra-, or pentamer) is controlled. This hypothesis of polymerization of *N*-acetylglucosamine subunits to form the oligosaccharide backbone is supported by the finding that the NodM protein has sequence homology with glucosamine synthase (encoded by *glmS*) (164) and therefore could have a function in the production of glucosamine, an obvious precursor of the synthesis of the oligosaccharide backbone. The predicted biochemical function of NodM is supported by the observation that the *nodM* gene was able to complement an *E. coli glmS* mutation (7). A mutation in *nodM* exhibits only a moderate delay in infection and nodulation (7, 255). This is consistent with the fact that a mutation in *nodM* results only in a quantitative decrease of the production of lipooligosaccharides (9, 243, 245). Leakiness might be due to the presence of a chromosomal counterpart of *nodM* (9, 164).

An important question is still which enzymes are responsible for the acylation of the chitin backbone. It is reasonable to assume that prior to the attachment of the correct fatty acyl chain to the nonreducing *N*-acetylglucosamine residue of the chitin backbone, the corresponding N-acetyl amino group must be deacetylated. Results from John et al. (125) indicate indeed that the NodB protein from *R. meliloti* deacetylates the nonreducing *N*-acetylglucosamine residue. Consequently, the last

TABLE 4. Some features of *nod* gene products

Nod protein	Species biovar[a]	Cellular location[b]	Sequence homology	Reference(s)[c]
NodA	Common	Cyt		6a, 126, 205
NodB	Common	Cyt	Deacetylase	6a, 125
NodC	Common	Inner mb	Chitin synthases	6, 13, 24, 48, 83
NodD	Common	Cyt mb	Transcription activator, LysR family	106, 222
NodE	Rl, Rt, Rm	Cyt mb	β-Ketoacyl synthase	17, 52, 246
NodF	Rl, Rt, Rm	Cyt	Acyl carrier protein	52, 81, 231
NodG	Rm		Alcohol dehydrogenase, β-ketoacyl reductase	49, 232
NodH	Rm		Sulfotransferase	204
NodIJ	Rl, Rt, Rm, Re, Ac, Bj, Bp	Cyt mb	Capsular polysaccharide secretion proteins	275
NolK	Bp			229
NodL	Rl, Rt, Rm, Bp	Cyt mb	Acetyltransferase	9, 18
NodM	Rl, Rt, Rm, Bp		D-Glucosamine synthase	8
NodN	Rl, Rt, Rm, Bp			8
NodO	Rl	Secreted	Hemolysin	258
NodP	Rm		ATP-sulfurylase	227
NodQ	Rm		ATP-sulfurylase and APS kinase	227
NodS	Re, Rtr, Rf, Bj, NGR, Ac		Methyltransferase (Ac)	80, 90, 152
NodT	Rl, Rt	Outer mb	Transit sequences	257
NodU	Re, Rtr, Rf, Bj, NGR, Ac			80, 90, 152
NodV	Bj	Cyt mb	Sensor, two-component regulatory family	89
NodW	Bj	Cyt	Regulator, two-component regulatory family	89
NodX	Rl*		Acidic exopolysaccharide encoded by *exoZ*	41, 72
NodY	Bj			182
NodZ	Bj			252
NolA	Bj		DNA-binding protein	211
NolB	Rf			170
NolC	Rf		Heat shock protein DnaJ	144
NolE	Rp	Secreted		42
NolFGHI	Rm			7
NolJ	Rf			20
NolK	Ac		Sugar epimerase	86
NolMNO	Bj			161
NolP	Rp			42
NolR	Rm		DNA-binding protein	140
NolT	Rf		*hrp* genes of pathogenic bacteria	170
NolUV NolW NolX	Rf			170
NolYZ	Bj			55

[a] Nod proteins are present in *R. leguminosarum* bv. viciae (Rl), *R. leguminosarum* bv. trifolii (Rt), *R. leguminosarum* bv. phaseoli (Rp), *R. meliloti* (Rm), *R. tropici* (Rtr), *R. etli* (Re), *R. fredii* (Rf), *Rhizobium* sp. strain NGR234 (NGR), *B. japonicum* (Bj), *Bradyrhizobium* sp. strain *Parasponia* (Bp), *A. caulinodans* (Ac), and *R. leguminosarum* TOM (Rl*).

[b] Cyt, cytoplasmic; mb, membrane.

[c] Only references that describe the most recent data about subcellular location, biochemical function, or homology are given.

step in forming the lipooligosaccharide backbone structure is the N-acylation on the deacetylated nonreducing end, which has been shown to be carried out by NodA (6a, 205).

Nothing is known about the transport of lipooligosaccharides out of the cell. The NodI protein shares sequence homology with a large family of traffic ATPases and has therefore been proposed to be involved in the secretion of the lipooligosaccharides (58, 275). However, transposon mutations in the *nodI* gene did not have a quantitative effect on the amount of lipooligosaccharides released in the growth medium (242). Recent results, however, with a multicopy plasmid containing cloned *nod* genes indicate that *nodI* and *nodJ* could have an influence on the excretion of lipooligosaccharides (241).

Synthesis of specific side chains. Several *nod* genes that were shown to be involved in the determination of the host range specificity of nodulation also appear to be involved in the production of lipooligosaccharides. Most noteworthy are the genes *nodQ* and *nodH*, which are the major determinants of host specificity in *R. meliloti* (75, 226, 227), and the gene *nodE*, which was shown to be the major determinant of host specificity in *R. leguminosarum* bv. viciae and bv. trifolii (58, 245).

The sequence homologies of NodF with the acyl carrier proteins and of NodE with various β-ketoacyl synthases (17, 58) lead to the hypothesis that these gene products function in the synthesis of the highly unsaturated lipid moiety. This hypothesis was further supported by the observation that NodF contains a 4′-phosphopantetheine prosthetic group (81), which can function as a carrier for acyl chains during fatty acid biosynthesis or transfer. The *nodE* mutants of both *R. leguminosarum* bv. viciae and *R. meliloti* secrete Nod metabolites that are N-acylated by vaccenic acid ($C_{18:1}$), the most common fatty acid in gram-negative bacteria. In contrast, the wild-type strains also secrete Nod factors with a highly unsaturated fatty acid, $C_{18:4}$ for *R. leguminosarum* bv. viciae and $C_{16:2}$ or $C_{16:3}$ for *R. meliloti* (52, 226, 245). This is explained in a simple model in which it is assumed that the NodE protein, by using regular fatty acid intermediates as substrates (which cannot be used by the normal household β-ketoacyl synthases), competes with normal fatty acid synthesis (82). In addition to Nod factors which are mono-N-acylated by unsaturated C_{16} fatty acids, *R. meliloti* produces a minor group of Nod factors which contain a series of C_{18} to C_{26} (ω-1)-hydroxylated fatty acids. The syn-

thesis of these N-acyl substituents is under control of the regulatory genes *nodD3* and *syrM* but is not specified by *R. meliloti nodEF* genes (51, 52). In *R. meliloti*, the *nodG* gene, which is not found in *R. leguminosarum*, resides downstream from *nodEF* (49, 77, 118). NodG shares homology with β-ketoacyl reductases and several alcohol dehydrogenases and might be involved in the synthesis of the acyl moiety (48, 77, 118). However, *nodG* mutations in *R. meliloti* did not result in a detectable change in the *R. meliloti* Nod factor acyl chain, but it is still possible that another *Rhizobium* gene can substitute for NodG function in the mutated strain (52).

In *R. meliloti*, a mutation in the *nodH* gene results in the absence of the sulfonation of all lipooligosaccharides. Mutations in *nodP* and *nodQ* genes result in the production of a mixture of sulfated and nonsulfated Nod factors (204). This leaky phenotype seems to result from the presence of a second copy of the *nodPQ* genes on the second megaplasmid (228). *R. meliloti* strains carrying mutations in both *nodPQ* copies do not produce sulfated factors (204). In gram-negative bacteria, the first steps of sulfate metabolism involve the synthesis of activated forms of sulfate derived from ATP, first the formation of adenosine 5′-phosphosulfate (APS) by ATP sulfurylase and second the formation of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) by APS kinase (142). The NodP protein and a region of NodQ share strong homology with CysD and CysN of *E. coli*, which are subunits of an ATP sulfurylase. Another region of *nodQ* is homologous to CysC, an APS kinase (228). In vitro studies have demonstrated that the NodP and NodQ proteins indeed have ATP sulfurylase and APS kinase activity (228), and their function is probably synthesis of PAPS. The NodH protein shares homology with sulfotransferases (204), which is consistent with its indispensable role in the addition of the sulfate moiety.

Mutations within *nodL* of *R. leguminosarum* strongly reduce nodulation on *Pisum*, *Lens*, and *Lathyrus* species but have only little effect on nodulation of *Vicia* species (256). *R. meliloti nodL* mutants exhibit only a slight delay of nodulation on alfalfa (53). NodL is homologous to bacterial acetyltransferases (8, 57), and in *R. leguminosarum* they specify the O acetylation on the C-6 of the nonreducing terminal glucosamine residue (18, 245). The same function is attributed to *R. meliloti nodL* (53).

Nod Metabolites: Variation on a Theme

Bradyrhizobium and *Azorhizobium* species are genetically quite distant from *Rhizobium* species (61, 124), but they were found to produce Nod metabolites belonging to the same family as those from *Rhizobium* (31, 172, 212).

Rhizobium sp. strain NGR234 produces a family of Nod metabolites which are also mono N-acylated pentamers of chitin with a variety of possible substitutions. The fact that NGR234 secretes a mixture of both sulfated and nonsulfated Nod metabolites may be part of the basis for its symbiotic promiscuity (192).

In *B. japonicum*, the Nod factors are also monoacylated chitin pentamers. However, the reducing end is substituted on C-6 with methylfucose (212). Results by Stacey et al. (252) show that the *nodZ* gene is responsible for the fucolysation of the core lipooligosaccharide nodulation signal. Since the Nod factors of *Rhizobium* sp. strain NGR234, also capable of soybean nodulation, have a 2-*O*-methylfucosyl (or fucose) residue (192), it suggests that this residue is critical for soybean nodulation. Indeed, the Nod factor isolated from a *B. japonicum nodZ* mutant is not able to induce cortical cell divisions on *Glycine soja* (252). However, the *nodZ* mutant is still able to nodulate most soybean genotypes (182, 252). Therefore, it is possible that the 2-*O*-methylfucose substituent is vital to soybean nodulation but that other functions in *B. japonicum* are able to compensate for the loss of this substituent (252). Also, mutations in the *nolO* gene of *B. japonicum* result in an alteration in the profile of nodulation signals, produced upon induction with flavonoids. Moreover, *nolO* mutants of *B. japonicum* produce a mixture of molecules with or without the 2-*O*-methylfucosyl residue (161). Because of the similarity between *nodZ*/*nolO* in *B. japonicum* and *nodH*/*nodPQ* in *R. meliloti*, Luka et al. (161) suggest that NodZ mediates the 2-*O*-methylfucosylation of the *B. japonicum* Nod metabolites utilizing a NolO-dependent substrate.

R. tropici strains produce a mixture of sulfated and nonsulfated Nod metabolites. Both stimulate external cortical cell division on *Phaseolus vulgaris*, but the sulfated compound is far more active. The Nod metabolites from *R. etli*, another symbiont of *Phaseolus vulgaris*, are quite different from those of *R. tropici*: they are nonsulfated and carry a modified fucose at the reducing end (165). Because of the major differences between the Nod factors of *R. tropici* and *R. etli*, Martinez et al. (165) suggest that *Phaseolus vulgaris* possesses different means to interact with Nod factors.

A. caulinodans also secretes Nod metabolites which are mono-N-acylated chitin oligomers, carrying several unusual substitutions (172). For example, an *N*-methyl group at the nonreducing end of all the molecules and the reducing glucosamine is substituted by a unique sugar, D-arabinose. Some of the *Azorhizobium nod* genes can be implicated in these modifications. The *nodS* gene most probably encodes an *S*-adenosylmethionine-dependent methyltransferase for Nod factor methylation (80), and a *nolK* gene may be involved in the synthesis of an arabinosyl precursor for factor glycosylation at the reducing end. These Nod metabolites have been found to elicit morphological changes on *Sesbania rostrata* roots: root hair deformation and induction of meristematic loci at lateral root bases, the sites where the root nodules are formed upon bacterial infection (112).

R. leguminosarum bv. viciae TOM can efficiently nodulate varieties of peas such as cv. Afghanistan (157). Strain TOM produces Nod factors that are similar to those of other *R. leguminosarum* bv. viciae strains. However, one of the nodulation factors made by strain TOM differs in that it carries an *O*-acetyl group on the C-6 of the reducing *N*-acetylglucosamine residue. This acetylation is NodX dependent. Although the *nodL* gene product is also an *O*-acetyltransferase, there is very little similarity between the derived amino acid sequences of the two acetyltransferases (72).

It thus appears that all rhizobia produce Nod metabolites which belong to the same family of molecules, lipooligosaccharides. The common core is probably synthesized by enzymes encoded by the common *nod* genes present in *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* species. The function of the host-specific *nod* genes is to code for enzymes which decorate the core molecules and make them plant specific.

ROLE OF NodO IN SYMBIOSIS

R. leguminosarum bv. viciae *nodE* mutants nodulate *Vicia* plants inefficiently (60). In the absence of the *nodE* gene, the host-specific nodulation factor with an acyl $C_{18:4}$ is not made and only the signal with an acyl $C_{18:1}$ group is formed. This latter signal cannot induce nodule meristem formation when added to *Vicia sativa* plants (245). Deletion analysis revealed that the *nodO* region was responsible for the reduced level of nodulation of peas or vetch observed with *nodE* mutants (60).

The NodO protein shows limited homology to secreted hemolysins and to some proteases (68) and shares a similar secretion mechanism to the secretion of hemolysin and protease (PrtB) by *Erwinia chrysanthemi* (216). Sutton et al. (258) recently showed that the *nodO* gene encodes a secreted protein with no detectable cellulase, pectinase, or protease activity but that it could form Ca^{2+}-regulated ion channels in an artificial membrane, similar to hemolysin-like toxins of certain mammalian pathogens. They concluded, since the pure lipooligosaccharides can induce nodule meristems in the absence of bacteria (245, 263), that NodO must have a complementary role to the nodulation factors. Such a role might be to contribute to the growth of the infection thread by causing a specific ion flux across the plasma membrane. With the recent finding of *nodO* in other rhizobia, such as *Rhizobium* sp. strain BR816 (271) and possibly also *B. japonicum*, this hypothesis can now be explored on a broader basis.

CONCLUSION

Establishment of the *Rhizobium*-plant symbiosis is the result of a multitude of communications between the symbionts and biochemical reactions within the symbionts. The study of mutants has shown that some of the steps can be uncoupled. A great majority of the knowledge is available on the mechanism of nodule formation. Less is known about the biochemistry of nodule function. Nevertheless, it should be realized that the knowledge about *Rhizobium*-plant symbiosis is based on a few selected systems, which might not cover the whole scale of *Rhizobium*-plant symbiosis occurring in nature. From these examples, a general mechanism can be formulated. With the characterization of new *Rhizobium* isolates, it is becoming clear that *Rhizobium* taxonomy requires constant updating. The question can be asked whether these new *Rhizobium* isolates, particularly from leguminous trees, will reveal other molecular mechanisms of nodule formation and/or infection.

In this review, little has been mentioned about plant genes that are essential for the *Rhizobium*-plant symbiosis, despite vast amounts of available information (for reviews, see references 50, 78, 108, and 276). Until now, these studies were based on the isolation and characterization of proteins and their transcripts that are specifically induced in nodule tissue. The proteins are called nodulins. Plant molecular biology now provides new tools to study nodule-specific genes, and it can be expected that application of these techniques (mutagenesis, transposon tagging, transformation, etc.) will shortly provide more knowledge in this area. Already, genetic linkage maps of legumes are being constructed and DNA sequencing techniques are being used to create physical maps of legume genomes (134).

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